PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 33/48, 33/53

A1

(11) International Publication Number: WO 94/17405

(43) International Publication Date: 4 August 1994 (04.08.94)

(21) International Application Number: PCT/US94/00455

(22) International Filing Date: 21 January 1994 (21.01.94)

(30) Priority Data:
007,432 22 January 1993 (22.01.93) US

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(71) Applicant: ADEZA BIOMEDICAL CORPORATION [US/US]; 1240 Elko Drive, Sunnyvale, CA 94089 (US).

(72) Inventors: LEAVITT, John, C.; 3400 Rambow, Palo Alto, CA 94306 (US). CASAL, David, C.; 574 Cutwater Lane, Foster City, CA 94404 (US). VARMA, Madhu; 154 Granada Drive, Mountain View, CA 94043 (US).

(74) Agents: TERLIZZI, Laura et al.; Skjerven, Morrill, MacPherson, Franklin & Friel, 25 Metro Drive, Suite 700, San Jose, CA 95110 (US).

(54) Title: ASSAY METHOD TO RULE OUT RUPTURE OF MEMBRANES IN WOMEN AT RISK FOR IMMINENT DELIVERY

(57) Abstract

The present invention provides an assay that distinguishes those patients with impending imminent delivery with intact membranes from those in whom the membranes have ruptured. The method comprises obtaining a cervicovaginal secretion sample from a pregnant patient determined to be as at risk for imminent delivery by detection of a biochemical marker for imminent delivery in a cervicovaginal secretion sample from the patient and determining the level of IGFBP-1 in the sample. If the level of IGFBP-1 is elevated, the patient has rupture of membranes. If IGFBP-1 is not present, the patient has intact membranes. In a preferred embodiment, the method comprises obtaining a cervicovaginal secretion sample from a pregnant patient after about week 20 of gestation and determining the level of fetal fibronectin and IGFBP-1 in the sample. The presence of an elevated fibronectin level in the sample indicates an increased risk of imminent delivery. If the level of IGFBP-1 is elevated, the patient had rupture of membranes. If IGFBP-1 is not present, the patient has intact membranes. If IGFBP-1 is not present, the IGFBP-1 assay is preferably repeated. In those patients with an increased level of IGFBP-1, the test indicates that delivery cannot be delayed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

ASSAY METHOD TO RULE OUT RUPTURE OF MEMBRANES IN WOMEN AT RISK FOR IMMINENT DELIVERY

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to methods for detection of impending delivery, and determining in those women at risk whether the membranes have ruptured. In particular, this invention is directed to the determination of impending delivery by detecting a biochemical marker for impending delivery in a cervicovaginal secretion sample and ruling out rupture of membranes as the cause of the risk by identifying a lack of IGFBP-1 in the sample.

Description of the Prior Art

Determination of impending preterm births is critical for increasing neonatal survival of preterm infants. In particular, preterm neonates account for more than half, and maybe as much as three-quarters of the morbidity and mortality of newborns without congenital anomalies. Although tocolytic agents which can delay delivery were introduced 20 to 30 years ago, there has been only a minor decrease in the incidence of preterm delivery. It has been postulated that the failure to observe a larger reduction in the incidence of preterm births is due to errors in the diagnosis of preterm labor and to the patients' conditions being too advanced for tocolytic agents to successfully delay the birth.

Traditional methods of diagnosis of preterm labor have high false-negative and false-positive error rates [Friedman et al, *Am. J. Obstet. Gynecol.*

104:544 (1969)]. In addition, traditional methods for determining impending preterm delivery, particularly in patients with clinically intact membranes, may require subjective interpretation, may require sophisticated training or equipment [Garl et al, *Obstet. Gynecol.* **60** :297 (1982)] or may be invasive [Atlay et al, *Am. J. Obstet. Gynecol.* **108**:933 (1970)]. An early, objective biochemical marker which indicated increased risk for preterm delivery was sought.

Fetal fibronectin is synthesized by extravillus trophoblasts as the trophoblasts invade the maternal decidualized uterus. Insoluble fetal fibronectin is laid down in the extracellular matrix of the placental bed. Soluble fetal fibronectin is found in amniotic fluid. The secretion of fetal fibronectin down the birth canal into cervical secretions may arise either by rupture of membranes when amniotic fluid is released down the birth canal or by release (solubilization) of fetal fibronectin from the extracellular matrix in the placental bed or release of fetal fibronectin from trophoblast cells. The presence of fetal fibronectin in cervical secretions is a predictor of preterm delivery.

Insulin-like growth factor binding protein one (IGFBP-1; other names for IGFBP-1 include pregnancy-associated endometrial α-globulin (αPEG), and placental protein-12 (pp 12) [Waites et al, *J. Clinical Endocrinology and Metab.* **67**:1100 1986]), which is synthesized by the uterine endometrium stroma in the late secretory pre-decidualization phase and pregnancy-induced maternal decidua in response to implantation of the fetus in the uterine wall, is an even more abundant protein of amniotic fluid than fetal fibronectin. IGFBP-1 appears to be efficiently transported into the amniotic sac from the decidua. The levels of IGFBP-1 in amniotic fluid

increase with the length of gestation. By the third trimester (27 weeks), IGFBP-1 levels increase to approximately 20 mg/ml, and IGFBP-1 becomes one of the most abundant proteins of amniotic fluid.

Lockwood et al [*New Engl. J. Med.*, **325**:669-674 (1991)] reported that fetal fibronectin in cervical and vaginal secretions indicates pregnancies which are at risk of imminent delivery. The authors postulate that damage to the fetal membranes may release fetal fibronectin into the cervix and vagina, giving rise to the biochemical marker. Fetal fibronectin is present in the secretions whether the impending delivery is due to rupture of membranes or to onset of labor.

Rutanen et al (*American Journal of Obstetrics and Gynecology* **164** (Supplement, Part 2):258, 1991) reported that IGFBP-1 was present in women with ruptured membranes and therefore at risk for imminent delivery. Like fetal fibronectin, IGFBP-1 is one of the most abundant proteins in amniotic fluid and is also present in the decidua, suggesting that IGFBP-1 may constitute another marker for impending delivery.

If IGFBP-1 or any other potential marker is released at a different point in the course of impending delivery, the marker could be used to evaluate the course of the disease.

SUMMARY OF THE INVENTION

The present invention provides an assay that distinguishes those patients with impending imminent delivery with intact membranes from those in whom the membranes have ruptured. The method comprises obtaining a cervicovaginal secretion sample from a pregnant patient determined to be as at risk for imminent delivery by detection of a biochemical marker for imminent delivery in a cervicovaginal secretion sample from the patient and determining the

level of IGFBP-1 in the sample. If the level of IGFBP-1 is elevated, the patient has rupture of membranes. If IGFBP-1 is not present, the patient has intact membranes.

In a preferred embodiment, the method comprises obtaining a cervicovaginal secretion sample from a pregnant patient after about week 20 of gestation and determining the level of fetal fibronectin and IGFBP-1 in the sample. The presence of an elevated fibronectin level in the sample indicates an increased risk of imminent delivery. If the level of IGFBP-1 is elevated, the patient had rupture of membranes. If IGFBP-1 is not present, the patient has intact membranes.

In a most preferred embodiment, the fetal fibronectin/IGFBP-1 test is preferably administered to women at about 20 weeks gestation and repeated at each antenatal visit (every two to four weeks) until at least week 37, preferably until delivery, if the test is negative. For those patients whose fetal fibronectin assay result indicates an increased risk of preterm delivery, the test of the patient's IGFBP-1 level determines whether the membranes are intact. If the test for IGFBP-1 is negative, the patient can be treated to prolong the pregnancy. The test of IGFBP-1 levels can be repeated during the course of treatment as often as daily to verify that the membranes remain intact. In those patients with an increased level of IGFBP-1, the test indicates that delivery cannot be delayed.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is an assay that rules out rupture of membranes in those patients with impending delivery, identifying those patients susceptible to treatments to delay delivery. The method comprises obtaining a cervicovaginal secretion

sample from a patient determined to be at risk for impending delivery by detection of a biochemical marker for impending delivery in a cervicovaginal secretion sample from the patient and testing a cervicovaginal secretion sample from the patient for IGFBP-1. If the level of IGFBP-1 is elevated, the patient has rupture of membranes. If IGFBP-1 is not detected, the patient has intact membranes. In a preferred embodiment, a cervicovaginal secretion sample from a pregnant patient is tested for both IGFBP-1 and the delivery marker. In a most preferred embodiment, the delivery marker is fetal fibronectin.

The invention also provides a kit comprising an anti-insulin-like growth factor binding protein one antibody and an antibody specific for an impending delivery marker, preferably fetal fibronectin.

Patients to be Tested

The present method can be used on any pregnant woman who may be at risk for impending delivery. In one embodiment of the method, an assay of IGFBP-1 is performed on a cervicovaginal secretion from a patient who has tested positive for the presence of a biochemical indicator of risk for impending delivery to determine whether the membranes are intact. In a preferred embodiment where both markers are assayed in the same sample, the method can be performed on any pregnant patient who satisfies the criteria for the impending delivery marker. For example, the presence of fetal fibronectin in cervicovaginal secretion samples is indicative of impending delivery after week 20 of gestation until delivery, whether delivery is early or post-term. Only patients in the appropriate gestational age range for fetal fibronectin should be tested for that combination of markers.

In addition to screening any woman to determine whether delivery is imminent, the patients who should be tested for a delivery indicator, are those patients with clinically intact membranes in a high risk category for preterm delivery, and preferably, all those women whose pregnancies are not sufficiently advanced to ensure delivery of a healthy fetus. Ninety percent of the fetal morbidity and 100 percent of the fetal mortality associated with preterm delivery is for those fetuses delivered prior to 32 to 34 weeks gestation. Therefore, 32 to 34 weeks gestation is an important cutoff for the health of the fetus, and women whose pregnancies are at least about 20 weeks and prior to 34 weeks in gestation should be tested.

In addition there are a large number of factors known to be associated with the risk of preterm delivery. Those factors include multiple fetus gestations; incompetent cervix; uterine anomalies; polyhydramnios; nulliparity; previous preterm rupture of membranes or preterm labor; preeclampsia; first trimester vaginal bleeding; little or no antenatal care; and symptoms such as abdominal pain, low backache, passage of cervical mucus and contractions. Any pregnant woman at 12 or more weeks gestation with clinically intact membranes and having one or more risk factors for preterm delivery should be tested throughout the risk period; i.e., until about week 37. Risk factors for spontaneous abortion include gross fetal anomalies, abnormal placental formation, uterine anomalies and maternal infectious disease, endocrine disorder cardiovascular renal hypertension, autoimmune and other immunologic disease, and malnutrition.

<u>Sample</u>

The sample is obtained in the vicinity of posterior fornix, the ectocervix or external cervical os. The sample generally comprises fluid and particulate solids, and may contain vaginal or cervical mucus and other vaginal or cervical secretions. Such samples are referred to herein and in the claims as cervicovaginal secretion samples. The sample is preferably removed with a swab having a dacron or other fibrous tip. Alternatively, the sample can be obtained with a suction or lavage device. Calculations to account for any additional dilution of the samples collected using liquids can be performed as part of the interpretation of the assay procedure.

Following collection, the sample is transferred to a suitable container for storage and transport to a testing laboratory. It is important that the sample be dispersed in a liquid which preserves proteinaceous analytes. The storage and transfer medium should minimize, preferably prevent, decline in the analyte level during storage and transport. A suitable solution for storage and transfer consists of 0.05 M Tris-HCl, pH 7.4; 0.15 M NaCl, 0.02% NaN_3, 1% bovine serum albumin (BSA), 500 Kallikrein Units/ml of aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM EDTA, and is described in U.S. Patent No. 4,919,889, issued April 24, 1990. The solution is also suitable as a sample diluent solution.

Alternatively, home and office use devices for immediate processing of the sample can be used. If used, the sample is placed directly in the device and testing is performed within minutes of sample collection. In such cases, the need to stabilize the analyte is minimized and any solution which

facilitates performing the assay and is not detrimental to analyte stability can be used.

Delivery Markers

Any biochemical marker of impending delivery which is assayed in a cervicovaginal secretion can be used in the present method. Suitable markers include, for example, elastase, total fibronectin, and fetal fibronectin. Most preferred is fetal fibronectin. Other markers which are predictive of impending delivery in cervicovaginal secretion samples are known and are useful in this method.

Elastase is an effective indicator of impending delivery in patients from about 12 weeks gestation to delivery. The marker is generally present in cervicovaginal secretion samples at levels about 30 units elastase per liter beginning about two to three weeks prior to delivery. Values less than 30 units per liter are considered negative.

Total fibronectin is an effective indicator of impending delivery in patients from about 12 weeks gestation to delivery. The marker is generally present in cervicovaginal secretion samples beginning about two to three weeks prior to delivery. The presence of an elevated level of total fibronectin is indicative of impending delivery. Preferably, the total fibronectin concentration in the sample is at least about 600 to 750 ng/ml of sample after week 20 of gestation. Between weeks 12 and 20 the threshold values vary. Values less than the specified threshold value are considered negative.

Fetal fibronectin is an effective indicator of impending delivery in patients from about 20 weeks gestation to delivery. The marker is generally present at levels about 50 ng/ml in cervicovaginal secretion samples beginning about one to two weeks prior to delivery.

Assay Procedure

When tested, the delivery marker is tested by any method that determines an increased risk of delivery. For example, a level of total fibronectin over a threshold value is indicative of impending delivery. However, a level of fetal fibronectin above background (e.g. the presence of fetal fibronectin) is indicative of impending delivery.

The following discussion is in terms of fetal fibronectin, as exemplary of the delivery marker, and IGFBP-1 as exemplary of how one determines a marker in a cervicovaginal secretion sample. IGFBP-1 is assayed by any quantitative or semi-quantitative procedure that can either determine the amount of IGFBP-1 in the sample or that the amount of IGFBP-1 is above a threshold amount that indicates rupture of membranes. For the delivery marker, the marker is assayed by any procedure that can either determine the amount of the marker in the sample or that the amount of the marker is above a threshold indicating imminent delivery.

Immunoassays are preferred. The antibody affinity required for detection of the analytes using a particular immunoassay method will not differ from that required to detect other polypeptide analytes. The antibody composition can be polyclonal or monoclonal. Anti-IGFBP-1 antibodies can be produced by a number of methods. Polyclonal antibodies can be induced by administering an immunogenic composition comprising human IGFBP-1 to a host animal. Alternatively, amniotic fluid or another source of high levels of IGFBP-1 can be used as the immunogen and antibodies of the desired specificity can be identified.

Preparation of immunogenic compositions of IGFBP-1 may vary depending on the host animal and is well known. For example, IGFBP-1 or an antigenic

portion thereof can be conjugated to an immunogenic substance such as KLH or BSA or provided in an adjuvant or the like. The induced antibodies can be tested to determine whether the composition is IGFBP-1-specific. If a polyclonal antibody composition does not provide the desired specificity, the antibodies can be purified to enhance specificity by a variety of conventional methods. For example, the composition can be purified to reduce binding to other substances by contacting the composition with IGFBP-1 affixed to a solid substrate. Those antibodies which bind to the substrate are retained. Purification techniques using antigens affixed to a variety of solid substrates such as affinity chromatography materials including Sephadex, Sepharose and the like are well known.

Monoclonal IGFBP-1-specific antibodies can also be prepared by conventional methods. A mouse can be injected with an immunogenic composition comprising IGFBP-1, and spleen cells obtained. Those spleen cells can be fused with a fusion partner to prepare hybridomas. Antibodies secreted by the hybridomas can be screened to select a hybridoma wherein the antibodies react with IGFBP-1 and exhibit substantially no reaction with the other proteins which may be present in a sample. Hybridomas that produce antibodies of the desired specificity are cultured by standard techniques. Hybridoma preparation techniques and culture methods are well known and constitute no part of the present invention.

Exemplary preparations of polyclonal and monoclonal antibodies is described in the examples. Antibody preparation and purification methods are described in a number of publications including Tijssen, P. Laboratory Techniques in Biochemistry and Molecular Biology: Practice and Theories of Enzyme

Immunoassays New York: Elsevier (1985), for example, and in the examples.

A number of different types of immunoassays are well known using a variety of protocols and labels. The assay conditions and reagents may be any of a variety found in the prior art. The assay may be heterogeneous or homogeneous, conveniently a sandwich assay.

The assay usually employs solid phase-affixed anti-IGFBP-1 antibodies. The antibodies may be polyclonal or monoclonal, preferably monoclonal. The solid phase-affixed antibodies are combined with the sample. Binding between the antibodies and sample can be determined in a number of ways. Complex formation can be determined by use of soluble antibodies specific for IGFBP-1. The antibodies can be labeled directly or can be detected using labeled second antibodies specific for the species of the soluble antibodies. Various labels include radionuclides, enzymes, fluorescers, colloidal metals or the like. Conveniently, the assay will be a quantitative enzyme-linked immunosorbent assay (ELISA) in which antibodies specific for IGFBP-1 are used as the solid phase-affixed and enzyme-labeled, soluble antibodies. Alternatively, the assay can be based on competitive inhibition, where IGFBP-1 in the sample competes with a known amount of IGFBP-1 for a predetermined amount of anti-IGFBP-1 antibody. For example, any IGFBP-1 present in the sample can compete with a known amount of the labeled IGFBP-1 or IGFBP-1 analogue for antibody binding sites. The amount of labeled IGFBP-1 affixed to the solid phase or remaining in solution can be determined.

Appropriate dilution of the conjugate can be performed to detect the selected threshold level of IGFBP-1 which is above background values for the assay as a positive sample.

Interpretation of Assay Result

IGFBP-1 levels below 20-50 ng/ml are considered background and are negative. The cut-off of choice for the background level depends upon whether a high sensitivity or high specificity test is desired. For example, as described in the examples, when 42 cervicovaginal secretion specimens which exhibited a positive fetal fibronectin test (> 50 ng/ml) for impending delivery and were ferning pooling and nitrazine negative for rupture of membranes were tested for IGFBP-1, one of these specimens demonstrated 42 ng/ml IGFBP-1. If a cut-off of 20 ng/ml were to be used, the demonstrated specificity of the test to rule out rupture would be 97%. On the other hand, if 50 ng/ml were to be used the rule out specificity of the test would be 100%. In most cases, high rule-out specificity would be the preferred method as patients with rupture of membranes are in greater danger of infection that those who do not have rupture, so the preferred cutoff is 20-50 ng/ml.

The cutoff of 20-50 ng/ml was determined for the assay described in the examples. As is well known, other assays using different reagents may have different cutoff values. For example, IGFBP-1 antibodies which differ in their antigen binding characteristics may produce assay results with different optimal cut off values. One of ordinary skill will recognize that background values may vary when different reagents are used and will understand how to determine the proper background level for the desired specificity and sensitivity for a selected assay.

The presence of IGFBP-1 in a cervicovaginal secretion sample from a patient who is positive for a marker that indicates increased risk of delivery indicates that the membranes have ruptured. If

IGFBP-1 is less than 20-50 ng/ml or undetectable (background for the assay), the membranes remain intact. When IGFBP-1 is positive (> 20-50 ng/ml) and the delivery marker is negative, then amniotic membranes may have ruptured, although most patients who have ruptured membranes will exhibit both positive IGFBP-1 and the delivery marker simultaneously.

As stated previously, the test can be administered to any pregnant woman who has tested positive for a marker that indicates increased risk of delivery. In a preferred embodiment, the delivery marker and IGFBP-1 are tested in the same cervicovaginal secretion sample. Such tests can be performed on any pregnant patient in the gestational age range for which the delivery marker is effective. Preferably, it is administered to all women with any known risk factor following 12 weeks gestation until delivery.

If the delivery marker test is negative, the woman is not at risk for impending delivery. The test can be repeated throughout gestation at regular antenatal visits or more frequently if the patient is high risk.

If the delivery marker test is positive (above the threshold value), the patient is tested for presence of IGFBP-1 in her cervicovaginal secretions. If IGFBP-1 is present in the secretions, the patient has ruptured membranes. If IGFBP-1 is negative, the membranes are intact.

When IGFBP-1 is negative, the IGFBP-1 test can be repeated, preferably daily, until the sample is positive for IGFBP-1. In addition, if a marker such as fibronectin which can be positive as much as weeks earlier than fetal fibronectin has been used, the marker which appears closest to delivery can be tested. If the delivery marker is positive and

IGFBP-1 is not present in the sample, measures to determine or enhance fetal lung maturity can be undertaken. For example, an amniotic fluid sample can be analyzed for phospholipids. In general, patients at risk for preterm delivery are examined every two weeks from about 22 to 36 weeks, rather than every four weeks as for patients in a low risk category. All patients are examined weekly from about week 36.

This invention is further illustrated by the following specific but non-limiting examples. Temperatures are given in degrees Centigrade and concentrations as weight percent unless otherwise specified. Procedures which are constructively reduced to practice are described in the present tense, and procedures which have been carried out in the laboratory are set forth in the past tense.

EXAMPLE 1

Quantitation of Fetal Fibronectin in a Vaginal Swab Sample

An immunoassay to determine fetal fibronectin in a vaginal sample used the reagents and procedures described below.

<u>Preparation of Monoclonal Anti-Fetal Fibronectin Antibody</u>

Preparation of the Hybridoma deposited at the American Type Culture Collection and given the accession number ATCC HB 9018 is described in detail in U.S. Patent No. 4,894,326 issued January 16, 1990 to Matsuura et al, which patent is incorporated herein by reference in its entirety.

The hybridoma was cultured by growth in RPMI 1640 tissue culture medium supplemented with 10%

fetal bovine serum. Additionally, the hybridoma was cultured in mice by the injection of the hybrid cells according to the procedure of Mishell and Shiigi (Selected Methods in Cellular Immunology, W.H. Freeman & Co, San Francisco p 368, 1980).

The monoclonal antibody designated FDC-6 and produced by the hybridoma was prepared for use in an immunoassay by the following procedure. The IgG fraction of the culture supernatant or the ascites was precipitated by ammonium sulfate fractionation. The antibody was redissolved and dialyzed into the appropriate buffer for purification by affinity chromatography on Protein-G Fast Flow (Pharmacia Fine Chemicals) according to the manufacturer's directions.

Preparation of Anti-Fetal Fibronectin Antibody-Coated Microtiter Plate

Microtiter plates were coated with FDC-6 monoclonal antibody by the procedure described below.

Monoclonal antibody FDC-6 prepared as described above was diluted to 10 μg/ml in phosphate buffer, pH 7.2 and 100 μl/well was dispersed into a polystyrene microtiter plate (Costar). The plates were incubated for 2 hours at room temperature or overnight at 4°C. The contents of the wells were aspirated and the wells washed 3 to 4 times with wash buffer (0.02 M Tris HCl, 0.15 M NaCl, 0.05% TWEEN-20). 200 μl/well of blocking/stabilizing solution (4% sucrose, 1% mannitol, 0.5% casein, 0.01 M PBS) was then added to the wells and incubated for 30 minutes to 4 hours at room temperature. The wells were then aspirated to dryness, and the plate was packaged in an air-tight container with a desiccant pouch, and stored at 4°C until needed.

The above procedure was repeated using microtiter plates from Nunc and Dynatech and gave equivalent results.

Preparation of Enzyme Labeled Anti-(Fibronectin) Antibody

Human plasma fibronectin was purified from human plasma as described by Engvall and Ruoslahti, *Int. J. Cancer* **20**:1-5 (1977). The anti-human plasma fibronectin antibodies were elicited in goats using the immunization techniques and schedules described in the literature, e.g., Stollar, *Meth. Enzym.* **70**:70 (1980), immunizing the goats with the human plasma fibronectin antigen. The antiserum was screened in a solid phase assay similar to that used for monoclonal antibodies, e.g., as described by Lange et al, *Clin. Exp. Immunol.* **25**:191 (1976) and Pisetsky et al, *J. Immun. Meth.* **41**:187 (1981).

The IgG fraction of the antiserum was purified further by affinity chromatography using CNBr-Sepharose 4B (Pharmacia Fine Chemicals) to which has been coupled human plasma fibronectin according to the method recommended by the manufacturer (AFFINITY CHROMATOGRAPHY, Pharmacia Fine Chemicals Catalogue 1990), pp 15-18.

Briefly, the column was equilibrated with from 2 to 3 volumes of buffer (0.01 M PBS, pH 7.2), and the anti-human fibronectin antibody-containing solution was then applied to the column. The absorbency of the effluent was monitored at 280 nm until protein no longer passed from the column. The column was then washed with equilibration buffer until a baseline absorbance at 280 nm was obtained.

The immunoaffinity bound anti-human plasma fibronectin antibody was eluted with 0.1 M glycine buffer, pH 2.5. Peak protein fractions were

collected, pooled and dialyzed against 0.01 M PBS, pH 7.2, for 24-36 hr at 4°C with multiple buffer changes.

The above procedure was repeated to immunize rabbits with human plasma fibronectin and to purify the resultant polyclonal anti-human fibronectin antibodies.

Anti-human plasma fibronectin antibody prepared as described above was conjugated with alkaline phosphatase following the one-step glutaraldehyde procedure of Avrameas, *Immunochem.* 6:43 (1969).

<u>Assay Reagents</u>

The assay was performed using the following additional reagents. The stock antibody conjugate was appropriately diluted in conjugate diluent (0.05 M Tris Buffer pH 7.2, 2% D-Sorbitol, 2% BSA, 0.1% Sodium Azide, 0.01% Tween-20, 1 mM Magnesium Chloride, and 0.1% Zinc Chloride) and 10 ml placed in a polyethylene dropper bottle container.

The enzyme substrate (10 ml in a polyethylene dropper bottle container) was phenolphthalein monophosphate (1 mg/ml) dissolved in 0.4 M aminomethylpropanediol buffer, pH 10 with 0.1 mM magnesium chloride and 0.2% sodium azide.

The positive control (2.5 ml in a polyethylene dropper bottle container) was amniotic fluid containing fetal fibronectin diluted to a concentration of fetal fibronectin of 50 ng/ml in sample diluent solution (0.05 M Tris buffer pH 7.4, 1 % BSA, 0.15 M sodium chloride, 0.02% Sodium Azide, 5 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 500 Kallikrein Units/ml of Aprotinin). This sample diluent solution is described in U.S. Patent No. 4,919,889 to Jones et al, issued April 24, 1990,

which patent is incorporated herein by reference in its entirety.

The negative control (2.5 ml in a polyethylene dropper bottle container) was the sample diluent solution used for the positive control without fetal fibronectin.

The rinse buffer (10 ml in a polyethylene dropper bottle container) was a 50X concentrate containing 1.0 M Tris buffer pH 7.4, 4.0 M sodium chloride, 2.5% Tween-20, and 1% sodium azide. The rinse buffer was diluted with water to a final concentration of 0.02 M Tris, 0.08 M sodium chloride, 0.05% Tween-20, and 0.02% sodium azide for use in the assay.

In addition, 5 μ pore size polyethylene sample filters (Porex Technologies, Fairburn, Georgia) were used to filter the samples prior to assay. All of the dropper bottles used to perform the assay were polyethylene bottles designed to dispense approximately 50 μl drops of the reagent. All of the assay steps performed following sample collection utilized the reagents and materials described above.

Assay Procedure

The assay was performed as follows. All samples were collected in the vicinity of the posterior fornix or cervical os using dacron swabs. Swab samples were immersed in 1.0 ml of sample diluent in a collection vial. The swabs were removed from the solution leaving as such liquid as possible in the collection tube. The samples were incubated at 37°C along with the controls for 15 minutes prior to the assay, either before or after filtration. A sample filter was snapped in place on each sample tube. Duplicate 100 μl aliquots of each sample and the positive and negative controls were placed in

separate wells of the microtiter plate and incubated for 1 hour at room temperature.

Following incubation, samples and controls were aspirated from the wells. Wells were washed three times with diluted wash buffer (1X). Following washing, 100 μl of enzyme-antibody conjugate was added to each well and incubated for 30 minutes at room temperature. The wells were aspirated and washed as described above. Following washing, 100 μl of enzyme substrate was added to each well and incubated for 30 minutes at room temperature.

Following the incubation, the plates were gently agitated by hand or with an orbital shaker to mix the well contents. The frame of strips was placed in an ELISA plate reader. The absorbance of each well at 550 nm was determined. The average absorbance of the duplicate wells for each sample and control was calculated. The fetal fibronectin concentration for the samples was calculated by preparing a standard curve and estimating that the samples were diluted to about one-tenth of their original concentration (collection of about 0.1 ml of sample combined with 1.0 ml of diluent). For this study, a cutoff of approximately 50 ng/ml was used as a positive sample. Samples below 50 ng/ml were considered to be background and negative.

EXAMPLE 2

Detection of IGFBP-1 in a Vaginal Swab Sample

IGFBP-1 was detected by the procedure described below.

<u>Preparation of Anti-IGFBP-1 Monoclonal Antibodies</u>

A panel of hybridomas was generated by immunization of mice with human amniotic fluid. One monoclonal antibody (designated AF127) reacted with a 31 kd protein which was found to be one of the most

abundant proteins of amniotic fluid. A two dimensional gel Western blot was used to identify the polypeptide antigen. This protein was so abundant in baboon amniotic fluid, that the protein was transferred to polyvinylidene difluoride membrane, which is a suitable absorbant for determining the sequence of a protein.

The N-terminus of the protein was sequenced using a single paper disk punched out with a conventional paper hole puncher containing 100 picomoles protein amino acid by Edman degradation amino acid sequencer using an Applied Biosystems, Inc. Model 477A amino acid sequencer. The N-terminal sequence was determined to be APWQCAPCSAEKLALCPPVPASCSEVTRSA, (SEQ ID NO. 1) which identified the protein as IGFBP-1 using GenBank.

The monoclonal antibody designated AF127 and produced by the hybridoma was prepared for use in an immunoassay by the following procedure. The IgG fraction of the culture supernatant or the ascites was purified using Avid Al affinity gel purification for immunoglobulins, according to the manufacturer's directions (Bioprobe International, Inc. Tustin, CA).

<u>Preparation of Anti-IGFBP-1-Coated Microtiter Plate</u>

Microtiter plates were coated with IGFBP-1 monoclonal antibody by the procedure described below.

Monoclonal antibody IGFBP-1 prepared as described above was diluted to 10 μg/ml in PBS (0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4, 0.02% NaN_3), and 100 μl/well was dispersed into a polystyrene microtiter plate (Costar). The plates were incubated overnight at 4°C. The contents of the wells were aspirated and the wells washed once with wash buffer (0.02 M Tris HCl, pH 7.9, 0.15 M NaCl). 250 μl/well of blocking solution (3% IGFBP-1-free BSA in PBS was then added to the wells and incubated for

2 hours at room temperature. The wells were aspirated and then washed once as described above and stored.

Preparation of Polyclonal Anti-IGFBP-1 Antibodies

IGFBP-1 was purified from baboon amniotic fluid using gel electrophoresis followed by electroelution/electrotransfer. The anti-baboon IGFBP-1 antibodies were elicited in goats using the standard immunization techniques and schedules, by immunizing the goats with the baboon amniotic fluid (which contained IGFBP-1).

The antiserum was screened in a solid phase assay similar to that used for monoclonal antibodies, e.g., as described by Lange et al, *Clin. Exp. Immunol.* **25**:191 (1976) and Pisetsky et al, *J. Immun. Meth.* **41**:187 (1981).

Assay Reagents

The assay was performed using the following additional reagents.

Commercially available swine anti-goat alkaline phosphatase antibody conjugate (TAGO, Burlingame, California) was appropriately diluted in conjugate diluent (0.02 M Tris buffer, pH 7.9, 1% BSA, 0.1% sodium azide, 0.05% TWEEN-20). The enzyme substrate was phenolphthalein monophosphate (1 mg/ml) dissolved in 0.4 M aminomethylpropanediol buffer, pH 10 with 0.1 mM $MgCl_2$ and 0.2% sodium azide.

The positive control was human amniotic fluid diluted to a concentration of IGFBP-1 of 50 ng/ml in sample diluent solution (0.02 M Tris buffer, pH 7.9, 0.5 % BSA, 0.15 M sodium chloride, 0.02% sodium azide.

The negative control was the sample diluent solution used for the positive control without IGFBP-1.

IGFBP-1 Assay Procedure

Cervicovaginal secretion samples were prepared as described in Example 1. Duplicate 100 μl aliquots of each sample or a dilution thereof, and the positive and negative controls were placed in separate wells of the microtiter plate and incubated for 2 hours at room temperature.

Following incubation, the wells were washed three times in rinse buffer (0.02 M Tris, pH 7.9, 0.15 M NaCl, 0.05% TWEEN-20, and 0.02% sodium azide)).

Following rinsing, 100 μl of goat anti-IGFBP-1 antibody (1:200 dilution) was added to each well and incubated for 2 hours at room temperature. Following the incubation, the plates were washed three times in rinse buffer. Following rinsing, 100 μl of swine anti-goat conjugate (1:4,000 dilution) was added to each well and incubated for 1 hour at room temperature. Following incubation, the plate was washed once in rinse buffer and 100 μl of enzyme substrate was added to each well. Kinetic absorbance values were read immediately at 405 nm using an ELISA plate reader. The plates were read again after half hour to determine the end point reading.

The average absorbance of the duplicate wells for each sample and control was calculated. The IGFBP-1 concentration for the samples was calculated by preparing a standard curve using amniotic fluid with known concentrations of IGFBP-1.

EXAMPLE 3

Study of a Panel of Patients

A panel of cervical secretion specimens from second and third trimester patients was tested for fetal fibronectin as described in Example 1. The panel was tested for rupture of membranes using

conventional ferning, pooling, and nitrazine. The same panel was then tested for IGFBP-1.

IGFBP-1 was not detectable (< 10 ng/ml) below 20 to 40 ng/ml in specimens that were negative for rupture of membranes by ferning, pooling, and nitrazine. Furthermore, IGFBP-1 was negative in specimens from women who were fetal fibronectin positive (> 50 ng/ml), rupture of membranes negative (by ferning, pooling, and nitrazine) and either pre-term delivery positive or negative as determined by outcome (whether the patient delivered at or before 37 weeks gestation). Most patients who were rupture of membranes positive by ferning, pooling, and nitrazine were also positive for IGFBP-1 (range 30 to > 5000 ng/ml).

The circulating levels of IGFBP-1 in maternal plasma were examined to determine if blood contamination of cervicovaginal secretions interfered with the test for IGFBP-1. The levels of IGFBP-1 in maternal plasma ranged from less than 10 ng/ml to 250 ng/ml and averaged about 150 ng/ml. Most of the rupture of membranes-positive cervicovaginal secretions specimens registered levels of IGFBP-1 of greater than 250 ng/ml. Thus, levels of IGFBP-1 in cervicovaginal secretions are a reliable indicator of rupture of membranes (ROM) in the presence of 10% blood or absence of blood in cervicovaginal secretion samples. Moreover, when fetal fibronectin is positive (> 50 ng/ml) the absence of IGFBP-1 is a reliable indicator that rupture of membranes has not occurred even though fetal fibronectin is present. The ability to rule-out rupture of membranes assists the physician in determining the approach to clinical management of the pregnancy.

EXAMPLE 4

Study of a Panel of Patients

In a second panel of patients, four groups of pregnant women were tested for fetal fibronectin and IGFBP-1 in cervical secretions. The groups were: (Group 1) those patients who were pre-term delivery positive (PTD+; delivery before 37 weeks) and fetal fibronectin positive (fFN+; > 50 ng/ml) but negative for rupture of membranes (ROM-) by ferning, pooling and nitrazine; (Group 2) those who were PTD- (delivery after 37 weeks) but who exhibited an fFN+ test (> 50 ng/ml) and were ROM- by ferning, pooling, and nitrazine; (Group 3) those who were PTD- (delivery after 37 weeks) and fFN- (< 50 ng/ml) and ROM- by ferning, pooling, and nitrazine; and (Group 4) those that were rupture of membranes positive (ROM+) by ferning pooling and nitrazine testing regardless of gestational age.

In Group 1, 23 out of 24 cervical secretion specimens exhibited less than 20 ng/ml IGFBP-1, while one specimen from a patient sampled at 27 weeks gestation exhibited 42 ng/ml IGFBP-1 in a specimen that also demonstrated greater than 1 microgram of fetal fibronectin per ml. Therefore, if the cut-off of the rupture of membranes (ROM) rule-out test was 50 ng IGFBP-1 per ml, the rule-out specificity (ROM rule-out specificity is the number of IGFBP-1 negative divided by the number of true ROM negative) would be 100% based upon ROM diagnosis by ferning, pooling, and nitrazine testing. If the cut-off were below 40 ng IGFBP-1 per ml the ROM rule-out specificity would be 96%.

In Group 2, 27 out of 27 cervical secretion specimens exhibited less than 20 ng/ml IGFBP-1. Thus, in this group, the rule-out of ROM was 100% specific based upon ROM diagnosis by ferning, pooling and nitrazine testing.

In Group 3, 23 out of 23 cervical secretion specimens exhibited less than 20 ng/ml IGFBP-1.

Thus, in this group, rule out of ROM was also 100% specific based upon ROM diagnosis by ferning, pooling and nitrazine testing.

In Group 4, 24 out of 30 specimens exhibited greater than 20 ng/ml IGFBP-1. Thus, in this group of patients who were diagnosed as ROM+ based upon ferning, pooling, and nitrazine testing, the IGFBP-1 test was 80% specific at diagnosing ROM. However, one of the six IGFBP-1 negative patients also exhibited a negative fetal fibronectin test (< 50 ng/ml) which should have been positive if amniotic fluid is present since fetal fibronectin present in amniotic fluid.

The finding that two abundant markers of amniotic fluid (IGFBP-1 and fetal fibronectin) gave results which were in disagreement with the results of the more subjective criteria of ferning, pooling, and nitrazine results calls into question the reliability of ferning, pooling and nitrazine testing for accurate diagnosis of rupture. Ferning, pooling, and nitrazine are well known to be a combination of tests which are inadequate for the determination of rupture. Specifically, when the test result is positive, amniotic fluid is likely to be present. However, a negative result may not indicate that amniotic fluid is absent, since the sensitivity of the test is low. However, since the test is subjective, both positive and results can be incorrect.

WHAT IS CLAIMED IS:

1. An immunoassay method for determining whether a pregnant patient determined to have impending preterm delivery by detection of a biochemical marker for impending delivery selected from the group consisting of elastase, total fibronectin, and fetal fibronectin in cervicovaginal secretions from the patient has intact fetal membranes, said immunoassay method comprising:
   a) obtaining a secretion sample from the vaginal cavity or the cervical canal from said patient; and
   b) determining the level of insulin-like growth factor binding protein one in the sample, the presence of insulin-like growth factor binding protein one above a predetermined level in the sample indicating rupture of fetal membranes.
2. The method of Claim 11 wherein the sample is removed from the posterior fornix.
3. The method of Claim 11 wherein the sample is obtained from the cervical os.
4. The method of Claim 11 wherein the biochemical marker for impending delivery is fetal fibronectin.
5. The method of Claim 11 wherein fetal fibronectin and insulin-like growth factor binding protein one are tested on the same sample.
6. The method of Claim 11 wherein the sample does not contain an elevated insulin-like growth factor binding protein one level and another sample from the patient is assayed for insulin-like growth factor binding protein one at least one day later.
7. The method of Claim 11 wherein a sample from the patient is obtained and assayed for the presence

of fetal fibronectin and insulin-like growth factor binding protein one at two week intervals after 20 weeks gestation until the patient reaches term or an elevated insulin-like growth factor binding protein one level is determined.

8. A method for determining impending delivery and rupture of membranes in a pregnant patient after 20 weeks gestation comprising:
   a. obtaining a secretion sample from the vaginal cavity or the cervical canal from said patient;
   b. determining the presence above a predetermined level of fetal fibronectin in the sample; and
   c. determining the presence above a predetermined level of insulin-like growth factor binding protein one in the sample,

   the presence of fetal fibronectin above the predetermined level in the sample indicating impending delivery; and the absence of insulin-like growth factor binding protein one above the predetermined level in the sample indicating intact fetal membranes.

9. An immunoassay kit comprising an anti-insulin-like growth factor binding protein one antibody and an antibody specific for an impending delivery marker selected from the group consisting of elastase, total fibronectin, and fetal fibronectin.

10. The immunoassay kit of Claim 9 wherein said an impending delivery marker is fetal fibronectin.

11. A method for determining whether a pregnant patient after 20 weeks gestation has impending delivery and intact membranes comprising:

a) obtaining a secretion sample from the vaginal cavity or the cervical canal from said patient;

b) determining the presence above a predetermined level of an impending delivery marker selected from the group consisting of elastase, total fibronectin, and fetal fibronectin in the sample; and

c) determining the presence above a predetermined level of insulin-like growth factor binding protein one in the sample,

the presence of the impending delivery marker above the predetermined level in the sample indicating impending delivery and the presence of insulin-like growth factor binding protein one below the predetermined level in the sample indicating intact fetal membranes.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00455

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/48, 33/53;
US CL :436/510, 65, 87;
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/510, 65, 87, 501, 503, 548, 63, 814; 435/975

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,096,830 (SENYEI ET AL) 17 MARCH 1992, see entire document, especially Col 1 and Col 4-5.	1-11
Y	EP, A, 0,316,919 (TENG ET AL) 24 MAY 1989, see entire document, especially pages 3, 10, and 21.	1-11
Y	NEW ENGLAND JOURNAL OF MEDICINE, Volume 325, Number 10, issued 05 September 1991, C.J. Lockwood et al, "Fetal Fibronectin in Cervical and Vaginal Secretions as a Predictor of Preterm Delivery", pages 669-674, see entire document, especially pages 669-671.	1-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 03 MARCH 1994	Date of mailing of the international search report APR 04 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer Jill Warden for JAMES L. GRUN, PH.D. Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 92/12426 (RUTANEN) 23 JULY 1992, see entire document.	1-11
Y	AM J OBSTET GYNECOL, Volume 164, Number 1 Part 2, issued January 1991, E. Rutanen et al, "Diagnosis of Premature Rupture of Fetal Membranes by the Measurement of Insulin-Like Growth Factor Binding Protein-1 in Cervical Secretion", page 258, Abstract No. 38, see entire document.	1-11
Y	JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM, Volume 67, Number 5, issued 1988, G.T. Waites et al, "Immunohistological Localization of the Human Endometrial Secretory Protein Pregnancy-Associated Endometrial α1-Globulin, an Insulin-Like Growth Factor-Binding Protein, During the Menstrual Cycle", pages 1100-1104, see especially pages 1101 and 1103.	1-11
Y,P	CLINICA CHIMICA ACTA, Volume 214, issued 1993, E. Rutanen et al, "Measurement of Insulin-Like Growth Factor Binding Protein-1 in Cervical/Vaginal Secretions: Comparison with the ROM-Check Membrane Immunoassay in the Diagnosis of Ruptured Fetal Membranes", pages 73-81, see entire document.	1-11

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG; search terms: insulin like growth factor (binding protein or receptor), insulin like(5w)binding, pregnan? endometri? globulin, placental protein, fibronectin, elastase, antibod?, Senyei, Rutanen;